1    **- Full title**

2    Death feigning as an adaptive anti-predator behavior: further evidence for its

3    evolution from artificial selection and natural populations

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5    **- Running title**

6    Death feigning as adaptive behavior

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24   **- Author contribution section**

25   The study was conceived by K.K., K.M., W.S., and T.M. The experiments were designed

26   by T.M., and these were performed by K.K., K.M., W.S., and K.M. The data was analyzed

27   by K.K., and K.M. The manuscript was written by K.K., and T.M. All authors approved

28   the final version prior to submission.

29

**Abstract**

Death feigning is considered to be an adaptive anti-predator behavior. Previous studies on *Tribolium castaneum* have shown that prey which death feign have a fitness advantage over those that do not when using a jumping spider as the predator. Whether these effects are repeatable across species or whether they can be seen in nature is, however, unknown. Therefore, the present study involved two experiments: 1) divergent artificial selection for the duration of death feigning using a related species *T. freemani* as prey and a predatory bug as predator, demonstrating that previous results are repeatable across both prey and predator species, and 2) comparison of the death-feigning duration of *T. castaneum* populations collected from field sites with and without predatory bugs. In the first experiment, *T. freemani* adults from established selection regimes with longer durations of death feigning had higher survival rates and longer latency to being preyed on when they were placed with predatory bugs than the adults from regimes selected for shorter durations of death feigning. As a result, the adaptive significance of death-feigning behavior was demonstrated in another prey-predator system. In the second experiment, wild *T. castaneum* beetles from populations with predators feigned death longer than wild beetles from predator- free populations. Combining the results from these two experiments with those from previous studies provided strong evidence that predators drive the evolution of longer death feigning.

54    **Main text**

55    **(1) Introduction**

56    Death-feigning (thanatosis or tonic immobility) is considered an adaptive strategy

57    exhibited by prey against predators (Miyatake et al., 2004; Ruxton et al., 2018). It is also

58    considered an adaptive behavior for females to avoid male harassment (e.g., Dennis and

59    Lavigne, 1976; Lawrence, 1992; Shreeve et al., 2006; Bilde et al., 2006; Khelifa, 2017) and

60    for individuals to avoid worker aggressions in social insects (van Veen et al., 1999). Although

61    the adaptive significance of death-feigning behavior has become widely recognized, it is still

62    partially in dispute due to a lack of evidence on what drives its evolution (See Honma et al.,

63    2006; Rogers and Simpson, 2018; Humphreys and Ruxton, 2018). Further, studies showing

64    survival benefits of death-feigning behavior along with estimates of heritability, i.e.,

65    evidence of the principal of natural selection (i.e., Darwin, 1859; Endler, 1986), have been

66    limited. An exception is previous work by Miyatake et al. (2004, 2009) which does artificial

67    selection for duration of death feigning over 10 generations in the red flour beetle, *Tribolium*

68    *castaneum*, and show that beetles with longer death-feigning duration are less likely to be

69    predated by a natural enemy, a jumping spiders, than beetles with shorter duration of death

70    feigning or no feigning death in laboratory experiments. However, the earlier studies did not

71    prove that death-feigning behavior was really advantageous in the presence of predators in

72    field conditions.

73    The present study involved two experiments: 1) divergent artificial selection for the

74    duration of death feigning using the Kashmir flour beetle, *Tribolium freemani*, as prey, to

75    investigate how selection on death feigning behaviour influences survival in the presence of

76    the predatory insect *Amphibolus venator,* and 2) comparison of the durations of death

77    feigning of *Tribolium castaneum* from field populations with and without the predatory bugs.

3

78    In the first experiment, we conducted divergent artificial selection to examine the three

79    prerequisites of natural selection: variation, fitness difference and inheritance (Endler,

80    1986) for death-feigning behavior using different prey and predator species than that used

81    previously (Miyatake et al., 2004) in order to provide cross-species replication. In the

82    present experiment, we established three replicated selection regimes for each direction of

83    a relatively short or long duration of death-feigning behavior and compared their fitness in

84    terms of survival in a predation test. In addition, we also compared the locomotor activity

85    of the selected regimes. This is because the previous studies in *T. castaneum* (Miyatake et al.,

86    2008; Matsumura et al., 2016), *T. confusum* (Nakayama et al., 2009), and another beetle

87    species, *Callosobruchus chinensis* (Nakayama and Miyatake, 2010), have observed a negative

88    genetic link between the death-feigning duration and locomotor activity, suggesting that

89    potential links with activity could constrain the evolution of death-feigning behavior.

90    In the second experiment, we compared the duration of death feigning in a related species,

91    *T. castaneum* beetles, from populations with and without predators. Previous studies

92    showed the duration of death-feigning behavior, which is controlled by brain dopamine

93    expression, is effective against enemies (e.g., Miyatake et al. 2004, 2008, 2009, Nakayama et

94    al. 2012, Uchiyama et al. 2019). However, we have not known if this predation pressure

95    actually works in nature.

96    We, therefore, hypothesized that our results from the two experiments (1) examining

97    survival rates of prey as different prey-predator species from the previous research and (2)

98    examining that wild beetles from populations with predators feigned death longer than wild

99    beetles from predator- free populations were combined, it would provide strong suggestion

100   that predators drive the evolution of longer death-feigning durations in nature.

101

## (2) Materials & Methods

2.1 Insects and culture

The Kashmir flour beetles (*Tribolium freemani* Hinton [Tenebrionidae]) used in this study were collected from a fiber warehouse located in Tokyo on 30 June 2014 (approximately 20 individuals) and maintained in an incubator (CR-41, Hitachi, Tokyo, Japan) kept at 29°C and under a photoperiod of 16 L:8 D. The beetles were reared in plastic Petri dishes (90 mm diameter and 15 mm height) with a mixture of wholemeal (Yoshikura-shokai, Tokyo, Japan) enriched with 5% brewer's yeast (Asahi Beer, Tokyo, Japan) as the rearing medium. We maintained the population size at about 100 to 200 larvae per 100 g of wheat and about 100 adults per container.

As a model predator, *Amphibolus venator* (Klug) [Reduviidae] was used in the study. This species is a predator of stored-product insect pests (Matsumra and Miyatake 2015). The body length of adult *A. venetaor* and *T. freemani* are about 10 mm and 5 mm, respectively. The population of this bug was collected from a rice bran storage warehouse in the city of Urasoe, Okinawa, Japan, in May 2015 and reared in plastic containers (30 mm diameter and 10 mm height) in an incubator (Sanyo, Osaka, Japan) maintained at 29°C and under a photoperiod of 16 L:8 D. Each bug was given three adult *T. castaneum* every week as food.

2.2. Experiment 1: artificial selection

*2.2.1 Artificial selection for death-feigning duration*

A random collection of 50 males and 50 females (14 days old) was made from the stock culture described above, and their death-feigning behavior was observed (F0 generation). Males and females (10 of each) with the shortest duration (short regimes) were selected to propagate short-duration lines; similarly, 10 of each with the longest duration were selected

126    to propagate long-duration lines (long regimes). The males and females of each line were

127    placed in a plastic cup (90 mm in diameter, 15 mm in height) with 20 g of medium and

128    allowed to lay eggs for one week. The pupae from the eggs were stored in separate-sex

129    groups in other plastic cups and allowed to emerge. Pupae were sexed based on the shape

130    of the abdominal end (Park 1934, Sokoloff 1974). When the adults reached 14 days old, 50

131    males and 50 females were randomly collected from each line and their death-feigning

132    duration was observed again (F1 generation). The same procedure was carried out in each

133    generation. Three selection replicates for the short and long lines (i.e., L1, L2, L3, S1, S2 and

134    S3) initiated at the same time were tested and maintained in the chamber. The selection

135    regimes were continued for 9 generations for each line. Realized heritability was calculated

136    as described by Falconer and Mackay (1996).

137    The observation method was the same as that used by Miyatake et al. (2004). That is,

138    twenty-four hours before the observation, each beetle was placed in one well of a 48-well

139    tissue culture plate (Cell Star, Greiner Bio-One, Kremsmünster, Austria) to avoid disturbance

140    by other beetles because it is known that contact between insects before stimulation

141    reduces the death-feigning duration (Miyatake, 2001). On the 14th day after eclosion, death-

142    feigning behavior was induced by touching the abdomen of a beetle with a wooden stick

143    with the same force throughout the experiment. A trial consisted of provoking the behavior

144    and recording its duration with a stopwatch. The duration was identified as the length of

145    time between touching the beetle and observing its first visible movement, such as

146    movement of a leg or antenna. All observations in this experiment were conducted by one

147    person (KK). In the previous studies with *T. castaneum* and *T. confusum* (Miyatake et al. 2004,

148    2009, Nakayama 2009), some individuals were unresponsive to the stimulus, and hence

149    sometimes we provided similar stimuli two more times, whereas all of the *T. freemani*

150 beetles feigned death at the first stimulus. All the trials were conducted between 12:00 and

151 18:00 in a chamber maintained at 25℃.

152 The previous selection experiments for duration of death feigning involved only two

153 replicate lines (*T. castaneum*, Miyatake et al., 2004; *Callosobruchus chinensis*, Ohno and

154 Miyatake, 2007, Nakayama and Miyatake, 2010). If artificial selection is performed for one

155 direction, the probability that the line will respond to the selected direction is 50%. If we

156 have two replicate lines with short and long durations, the probability that all lines will

157 respond to the selected direction is 0.0625, which is still over 5%. Therefore, the possibility

158 of genetic drift could not be rejected completely in those studies. Thus, selection

159 experiments must include three replicated lines for each divergent direction in cases

160 without control strains because the probability is 0.015625 ($P<0.05$) in the case with three

161 replicated lines for each direction.

162

163 *2.2.2 Predation tests*

164 Virgin adults of *Amphibolus venator* were used as model predators. In Japan, *A. venator* has

165 been found in a rice bran storage warehouses inhabited by many *T. castaneum*, but no *T.*

166 *freemani* (TM, unpublished data). The numbers of males and females were 12 and 12,

167 respectively (N=24). Before the test, each predatory insect was starved for 10 days. All

168 predators encountered a prey only once, and thus each bug was tested with an adult beetle

169 from each selection regime and each replicate (2 regimes selected x 3 replications within

170 each selection line x 24 bugs = 144 matches in total). We also performed statistical analyses

171 on the effect of the order in which predators encounter their prey, i.e., order of predation, as

172 described later.

173 Each *A. venator* individual was gently placed on a Petri dish (60 mm in diameter, 15 mm in

174    height) lined with filter paper and covered by a smaller Petri dish (30 mm in diameter, 15

175    mm in height). Then, a *T. freemani* virgin adult male derived from either the long or the short

176    selection regime (7 days after eclosion, from the 8th generation) was introduced into the

177    Petri dish, because previous studies using related species indicated no sexual difference in

178    death-feigning duration in *T. castaneum* (Miyatake et al. 2004, 2008) and *T. confusum*

179    (Nakayama et al. 2009). Each individual was kept in smaller Petri dish for 5 min before the

180    experiment to adapt to the environment. After 5 min, we removed the smaller Petri dish and

181    then observed predation. The period from the start of the test until successful predation

182    occurred was timed with a stopwatch, and observation was carried out for up to 5,000 sec

183    (ca. 83.3 min). Trials in which individuals survived predation for 5,000 sec were recorded

184    as non-predation trials. All experiments were carried out between 12:00 and 18:00 in a

185    chamber maintained at 25°C with a 16-hour photoperiod (lights on at 07:00, lights off at

186    23:00).

187

188    *2.2.3 Locomotor activity*

189    The locomotor activity of each beetle (7–21 days old, virgin) from the short and long

190    selection regimes ($N$ = 585) was monitored using an infrared actograph. Each beetle was

191    placed into a Petri dish (30 mm in diameter, 15 mm in height) without food. Locomotor

192    activity was measured by an infrared light beam which passed through the clear petri dish

193    and projected onto a photomicrosensor (E3S-AT11; Omron, Kyoto, Japan) that detected all

194    interruptions of the light beam. Interruptions of the infrared light beam were recorded

195    every 6 min. After the beetles were put into the Petri dish, we measured the locomotor

196    activity for 24 h. To avoid the effects of stimuli from humans, the data from the first 2 h of

197    measurement were removed (i.e., data from 22 h were used for statistical analysis). All

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198    experiments were carried out in an incubator maintained at 25°C under dark conditions.

199

200    *2.2.4 Body mass*

201    A previous study showed a positive correlation between death-feigning duration

202    and body weight in *Callosobruchus chinensis* (Hozumi and Miyatake 2005), which

203    might relate to body size balance between prey and predators. Moreover, the risk of

204    predation and locomotor activity may also be affected by body mass. Therefore, we

205    compared the body mass of *T. freemani* adults from each selection regime. Ten males

206    and 10 females were randomly collected from each line (i.e. 10 × sex × selection regime ×

207    replicate line = 120), and the body mass of each beetle was measured by an electronic

208    balance to 0.001mg (BP 211 D, Sartorius AG, Göttingen, Germany).

209    Although, we did not measure body sizes of each predator and prey in the present study,

210    each beetle was randomly used from long and short selection regimes, respectively. We thus

211    considered that the effect of a difference in body sizes of predator and prey for predation

212    experiments was relatively small.

213

214    2.3 Experiment 2: comparison of natural populations

215    We used two populations of *T. castaneum*. One is the Base population, which has been

216    reared for more than 20 years in our laboratory without any predator in an incubator

217    maintained at 25°C under a photoperiod of 16 L:8 D (see Miyatake et al., 2004). Another *T.*

218    *castaneum* population, which has lived together with *A. venator* for more than 20 years in

219    the same rice bran storage warehouse described above (Okinawa population; T.M.,

220    unpublished), was collected from the warehouse in May 2015, and the population was

221    reared in the laboratory until the present experiment was conducted under the same

222 conditions as those for the Base population. *A. venator* individuals have been found only in

223 a rice bran storage warehouse in Japan, and thus we did not have another replicate with a

224 similar environment. The procedures for death-feigning behavior observation were the

225 same as described above.

226

227 2.4 Statistical analysis

228     The realized heritability of the artificial selection for death-feigning behavior duration

229 in *T. freemani* was calculated according to the regression of the selection responses on the

230 cumulative selection differential for the first 9 generations (Falconer and Mackay, 1996). To

231 test for the realized heritability, we used analysis of variance (ANOVA) for each line of

232 selection regimes. To test for the direct response to selection, the duration of death-feigning

233 behavior in the 9th generation was analyzed by a generalized linear model (GLM) with a

234 gamma distribution and selection regime, sex, and interaction between selection regime and

235 sex as explanatory variables, and the replicate line of each regime was nested within the

236 selection regime.

237     In the analysis of the latency of predation by *A. venator*, we used a survival analysis with

238 Cox proportional hazards regression and a likelihood ratio test, because we had set an upper

239 limit to the time data (5000 s). In this analysis, selection regime, sex, order of predation

240 (each bug was used 5 times in the predation experiment), and interaction between selection

241 regime and sex as explanatory, and the replicate line was nested within the selection regime.

242 To test the predation rate (i.e., the proportion of individuals that survived over 5000 s), a

243 GLM with a binomial distribution was used, with selection regime, sex, order of predation,

244 and interaction between selection regime and sex as explanatory variables, and the replicate

245 line was nested within the regime. A GLM with a gamma distribution and selection regime,

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246 sex, and interaction between selection regime and sex as explanatory variables was used for

247 tests of the locomotor activity of beetles from each selection regime. In this analysis, the

248 replicate line was nested within the selection regime.

249     Body mass was analyzed by nested ANOVA with selection regime, sex, and interaction

250 between regime and sex as explanatory variables; the replicate line was nested within the

251 selection regime.

252     To compare the duration of death-feigning behavior between populations with and

253 without predators in *T. castaneum*, we used a GLM with a gamma distribution and

254 population and sex as explanatory variables.

255     All analyses were conducted in R version 3.4.3 (R Core Team, 2017).

256

257 **(3) Results**

258 3.1 Experiment 1: artificial selection

259     The direct responses to selection for death-feigning duration in males and females are

260 shown in Fig. 1, and the realized heritability in each line is shown in Table 1. For generation

261 6 of replicates A (short selection regime) and B (long selection regime), data were lost as a

262 result of an accident. However, the long strains showed clear responses to selection and

263 significantly high heritability, whereas the short strains showed no clear responses to

264 selection (Fig.1, Table 1).

265     In the 9th generation, beetles from the long selection regimes showed a significantly

266 longer duration of death-feigning behavior than the short selection regimes (Table 2).

267 Replicate lines nested within the selection regime showed significant effects on the death-

268 feigning duration (Table 2). Sex and interaction between selection regime and sex were not

269 shown to significantly affect the duration of death feigning (Table 2).

11

270    Beetles from the short selection regimes showed a significantly shorter latency to

271    predation and a higher rate of predation by *A. venator* than those from the long selection

272    regimes (Fig. 2, Table 3). Replicate lines nested within the selection regime showed

273    significant effects on the latency to predation (Table 3). There was no significant effect of

274    order, sex, or interaction between selection regime and sex on the latency and rate of

275    predation (Table 3).

276    Locomotor activity as a correlated response to selection showed that beetles from long

277    selection regimes showed significantly lower locomotor activity than short selection

278    regimes (Fig. 3, Table 4). Males showed significantly higher locomotor activity than females

279    (Table 4). Replicate lines nested within the selection regime showed significant effects on

280    the locomotor activity (Table 4). There were significant effects of interaction between

281    selection regime and sex in locomotor activity (Table 4).

282    Beetles from the long selection regime were significantly heavier than those from the

283    short selection regime (Fig. 4, Table 5). Females were heavier than males (Table 5). Replicate

284    lines nested within the selection regime showed significant effects on the body mass (Table

285    5). There was no significant effect of interaction between selection regime and sex on body

286    mass (Table 5).

287

288    3.2 Experiment 2: comparison of natural populations

289    In *T. castaneum*, beetles from the natural population (i.e., the population from Okinawa)

290    showed a significantly longer duration of death feigning than the predator-free populations

291    (i.e., base lab population) (Fig. 5, Table 6). There were no significant effects of sex or the

292    interaction between population and sex (Table 6).

293

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**(4) Discussion**

The artificial selection experiment and population comparison presented here, provide evidence for heritable variation in death-feigning behavior as well as evidence for it's role in deterring predation and increasing survival. Combined with previous studies showing similar results in other species (Miyatake et al., 2004, 2009; Nakayama et al., 2010), our results suggest that these patterns can be generalized across species.

In the first experiment, divergent regimes selected for the duration of death feigning were successfully established for *T. freemani*, resulting in a 6-fold difference between the short- and long-duration selection regimes in each replicate (Fig. 1). Highly significant realized heritability was shown for the selection for a long duration of death feigning, while only some significant heritability was identified in the short-duration selection regimes (Table 1).

Although the predator used in the previous study was a jumping spider (Miyatake et al., 2004), a different predator, a predatory insect, was used in the present experiment. The predator used in this study is more common in storage houses than the jumping spider (TM, personal observation in some places), although both are natural predators. Since both predators are of the type to attack in response to a moving prey, death-feigning behavior of the prey would be effective against this type of predator in spite of the differences in taxa, i.e., jumping spiders and predatory bugs. Thus, it is suggested that the strategy of immobility may frequently evolve in the relationship between prey and predator invertebrate system in the field (see Miyatake et al., 2004; Ruxton et al., 2018). This may be a reason why death-feigning behavior is found in very broad taxa including mammals, birds, fishes, amphibians, reptiles, mites, spiders, and especially insects (Miyatake et al. 2004, 2009), although different patterns of adaptability of prey immobility can also evolve, such as a physical

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318    defense against being swallowed by predatory frogs (Honma et al. 2006).

319    Negatively correlated responses of locomotor activity to selection for the duration of

320    death feigning were found in the three artificially selected replicates (Table 2), which is

321    similar to what has been observed in other species examined: *T. castaneum* (Miyatake et al.,

322    2008; Matsumura et al., 2016), *T. confusum* (Nakayama et al., 2009), and another beetle

323    species, *Callosobruchus chinensis* (Nakayama and Miyatake, 2010). This may show that

324    potential links with locomotor activity could constrain the evolution of death-feigning

325    behavior. Further, it may demonstrate the dopaminergic system as a mechanism underlying

326    personality, because higher brain dopamine expression was found in regimes selected for

327    shorter than longer duration of death feigning in *T. castaneum* (Miyatake et al. 2008; Nishi

328    et al. 2010) and in *T. confusum* (Nakayama et al. 2012). Recently, Uchiyama et al. (2019)

329    found different expressions of genes between the regimes selected for longer and shorter

330    duration of death feigning in dopamine-related genes in the tyrosine metabolism pathway

331    of *T. castaneum* by transcriptomic comparison between the beetle selection regimes.

332    Therefore, comparison of expressions of genes between the selection regimes in *T. freemani*

333    would be interesting in the future.

334    In the present study, significant interaction effects between selection regime and replicate

335    were found (Table 2), and this result differs from those found in previous selection

336    experiments for death-feigning duration with no significant effects between them (e.g.,

337    Miyatake et al., 2004, 2008, Nakayama et al., 2009). In *T. castaneum*, we used walking

338    distance measured using a color tracker as an index of insect activity (Miyatake et al., 2008).

339    On the other hand, each insect was placed in a tube, and locomotor activity was measured

340    as activity in the case of *T. confusum* (Nakayama et al., 2010). In all of the previous

341    experiments, the durations of death feigning were negatively correlated with the activity

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342    level; a shorter duration of death feigning was linked with higher activity, and no interaction

343    was found between selection regimes and replicates (Miyatake et al., 2008, Nakayama et al.,

344    2010). However, the present results showed a significant interaction effect, although the

345    reason is unknown. Therefore, it is necessary to investigate interactions among activity-

346    related traits in the future.

347    In the present study, a larger body mass was found in beetles derived from long rather

348    than short selection regimes (Figure 4, Table 5). In some cases, larger beetles could be

349    assumed to be at higher risk of being caught by a predator because they are big and easy to

350    find, while smaller beetles could be also assumed to have a higher risk because of higher

351    mobility. *T. freemani* from the long selection regime showed a significantly lower risk of

352    predation and lower locomotor activity. It suggests that the short strain is preyed on more

353    easily not because it remains immobile for a shorter time, but simply because it is smaller.

354    For example, in spider species, the prey that is larger relative to the predator was less likely

355    to be caught by a predatory spider (Michalek et al. 2019). On the other hand, there is the

356    argument that a large body may be easy for enemies to find. For example, in a giant clam

357    *Tridacna maxima*, larger clams were eaten by predators more often than smaller clams

358    (Johnson et al. 2017). Therefore, the relationships may depend on the type of predator, and

359    it is required to examine the relationship between relative prey-predator sizes and the

360    efficiency of death-feigning tactics in the future.

361    Hozumi and Miyatake (2005) reported a positive correlation between death-feigning

362    duration and body mass in *C. chinensis*, in accordance with the present result. Because

363    smaller individuals in *C. chinensis* have shorter longevity, it was assumed that they invest

364    more in reproduction rather than in anti-predator behavior (Hozumi and Miyatake 2005).

365    Therefore, because the investment strategies may be genetically correlated with the body

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366  size, body size may positively respond to artificial selection for duration of death-feigning in

367  *T. freemani* as well. We need additional study that investigates the relationship between

368  lifespan and reproductive success in long and short selection regimes in *T. freemani* in the

369  future.

370  In the present study, we did not measure the body size of each predator. However, each bug

371  was randomly used as long and short beetles, respectively. We, therefore, considered that

372  the effect of body size of predators in the predation experiment was relatively small.

373  Why was the duration of death feigning in *T. freemani* longer than that of *T. castaneum* and

374  *T. confusum*? Although we did not compare *T. castaneum* by statistical methods, the mean

375  duration of death feigning was approximately 60 sec, and it became longer, reaching 120 sec,

376  after 10 generations of artificial selection for a longer duration of death feigning (Miyatake

377  et al., 2004). In *T. confusum*, the death-feigning duration was approximately 10 sec in the

378  population before the selection experiment, and it became longer (from 100 to 200 sec

379  depending on the selected lines) after 17 successive generations of selection for the duration

380  of death feigning (Nakayama et al., 2010). On the other hand, the death-feigning duration of

381  *T. freemani* was already approximately 500 sec before the artificial selection was carried out

382  (see Fig. 1). The death-feigning duration lengthened to 3,000 sec after 9 generations of

383  artificial selection for longer duration of death feigning in *T. freemani*, while regimes selected

384  for a shorter duration did not respond; thus, the duration of death feigning is still 500 sec

385  (see Fig. 1). These results suggest large interspecific variation in the duration of death

386  feigning, even in the genus *Tribolium*. *T. freemani* originally had much longer death-feigning

387  duration and low genetic variation in shorter duration of death feigning, resulting in low

388  heritability in short-duration selection regimes (see Table 1).

389  We propose here the following two hypotheses for the longer duration of death feigning

16

390      in *T. freemani* than that in other *Tribolium* species. The first is the difference in body size of

391      these species: adults of *T. freemani* (body length: approximately 5 mm) are larger than those

392      of *T. castaneum* (from 3 to 4 mm) and *T. confusum* (from 3 to 4 mm). In the other beetle, *C.*

393      *chinensis*, a larger body size was positively correlated with a longer duration of death

394      feigning within the species (Hozumi and Miyatake, 2005), suggesting that small individuals

395      did not have a greater ability to feign death than larger ones, as described above. The second

396      reason is a bottleneck. The *T. freemani* population used in the present study originated from

397      approximately 20 adults collected from one place in Tokyo. Therefore, the genetic alleles of

398      original population could have been fixed for a relatively long duration of death feigning.

399      More importantly, here we compared the duration of death feigning of a population

400      collected from a field with *A. venator* and another population reared in the laboratory

401      without the predator of *T. castaneum*. Beetles raised with the predator showed longer

402      duration of death feigning than beetles raised without the predator. The two results,

403      experimental evolution (artificial selection) and the comparison of beetle populations

404      collected from fields with and without the predatory bug may suggest more strongly the

405      evolution of death-feigning behavior in nature.

406      All the results strongly support the theory that death feigning is adaptive and evolvable

407      as an anti-predator strategy even under natural conditions like stored warehouse where

408      predators and prey live together for generations. The present results also provide another

409      example of the heritable variation in fitness associated with death-feigning behavior in

410      addition to the results from the previous study (Miyatake et al., 2004). However, as

411      mentioned in Materials and Methods, *A. venator* individuals have been found only in a rice

412      bran storage warehouse in Japan, and thus we did not have another replicate with a similar

413      environment. In the future, it is necessary to examine the death-feigning behavior of

414     *Tribolium beetles* at multiple locations where predators are present.

415     In conclusion, we were able to demonstrate that variation in adaptive fitness traits

416     associated with the survival rate was strictly greater in the death-feigning duration of *T.*

417     *freemani* as well as *T. castaneum*. This series of artificial selection experiments suggests the

418     necessity of verification to examine the relationship between death feigning and activity-

419     related traits. Additionally, interspecific variation in the duration of death feigning was

420     found, and the reason for this variation needs to be examined further in the future. When

421     we combined the two results for experimental evolution (artificial selection) and

422     comparison of natural populations, we can assert more strongly that the presence of

423     predatory insects can lead to evolution of longer death feigning in nature.

424

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498

499    **Table 1**. Realized heritability ($h^2$) of the duration of death feigning in each selection regime.

| Selection regime | Replicate | $h^2$ | | | |
|---|---|---|---|---|---|
| | | Male | | Female | |
| Long | 1 | 0.112 | ** | 0.171 | ** |
| | 2 | 0.150 | ** | 0.189 | ** |
| | 3 | 0.162 | *** | 0.213 | *** |
| Short | 1 | -0.037 | | -0.005 | |
| | 2 | -0.094 | * | -0.094 | * |
| | 3 | -0.081 | * | -0.047 | |

500    *$P<0.05$

501    **$P<0.001$

502    ***$P<0.0001$

503

22

504 **Table 2**. Results of the GLM for duration of death feigning in the 9th generation.

| Factor | d.f. | $\chi^2$ | p |
|---|---|---|---|
| Selection regime | 1 | 789.19 | < 0.0001 |
| Replicate line [selection regime] | 4 | 62.72 | < 0.0001 |
| Sex | 1 | 0.70 | 0.4021 |
| Selection regime*sex | 1 | 0.16 | 0.6851 |
| Error | 498 | | |

505

506

507 **Table 3**. Results of the GLM for latency and rate of predation.

| Trait | Factor | d.f. | $\chi^2$ | p |
|---|---|---|---|---|
| Latency | Selection regime | 1 | 30.48 | <0.0001 |
| | Replicate line [selection regime] | 4 | 12.15 | 0.0163 |
| | Sex | 1 | 1.39 | 0.2390 |
| | Selection regime*sex | 1 | 0.86 | 0.3541 |
| | Order of predator | 1 | 0.45 | 0.5031 |
| | Error | 135 | | |
| Rate | Selection regime | 1 | 23.06 | <0.0001 |
| | Replicate line [selection regime] | 4 | 7.56 | 0.1090 |
| | Sex | 1 | 1.1 | 0.2937 |
| | Selection regime*sex | 1 | 0.53 | 0.4665 |
| | Order of predator | 1 | 0.89 | 0.3459 |
| | Error | 135 | | |

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509

510 **Table 4**. Results of the GLM for locomotor activity in *T. freemani.*

| Factor | d.f. | $\chi^2$ | p |
|---|---|---|---|
| Selection regime | 1 | 12653.08 | <0.0001 |
| Replicate line [Selection regime] | 4 | 10557.44 | <0.0001 |
| Sex | 1 | 2410.36 | <0.0001 |
| Selection regime*Sex | 1 | 7.29 | 0.0069 |
| Error | 577 | | |

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513 Table 5. Results of the nested ANOVA for body mass in *T. freemani*.

| Factor | d.f. | F | p |
|---|---|---|---|
| Selection regime | 1 | 6.14 | 0.0147 |
| Replicate line [Selection regime] | 4 | 5.84 | 0.0003 |
| Sex | 1 | 7.00 | 0.0093 |
| Selection regime*sex | 1 | 0.00 | 0.9723 |
| Error | 108 | | |

514

515

516 **Table 6**. Results of the GLM analysis of duration of death feigning in *T. castaneum* beetles

517 from the Base and Okinawa populations.

| Factor | d.f. | $\chi^2$ | P | |
|---|---|---|---|---|
| Population | 1 | 127.34 | | <0.0001 |
| Sex | 1 | 0.37 | | 0.5425 |
| Population*sex | 1 | 0.06 | | 0.8114 |
| Error | 292 | | | |

518
519

520   Figure legends

521

522   **Figure 1**. Direct responses to artificial selection for the duration of death feigning in the long

523   (filled symbols) and short duration (open symbols) treatments in *T. freemani*. Circles,

524   squares, and rhombuses represent replicate lines 1, 2, and 3, respectively. Error bars show

525   SE.

526

527   **Figure 2**. Latency of predation in selection regimes (results of selection in the twelfth

528   generation). Each bar shows replicate lines (black: 1, white: 2, gray: 3). Error bars show SE.

529   Predation rates (percentages) are shown above the error bars.

530

531   **Figure 3.** Response of locomotor activity to artificial selection for the duration of death

532   feigning. Each bar shows replicate lines (black: 1, white: 2, gray: 3). Error bars show SE.

533

534   **Figure 4**. Body mass in selection regimes. Each bar shows replicate lines (black: 1, white: 2,

535   gray: 3). Error bars show SE.

536

537   **Figure 5**. Comparison of the duration of death feigning in beetles from the Base (without

538   predator condition) and Okinawa (with predator condition) populations of *T. castaneum*.

539   Black and white represent males and females, respectively. Error bars show SE.
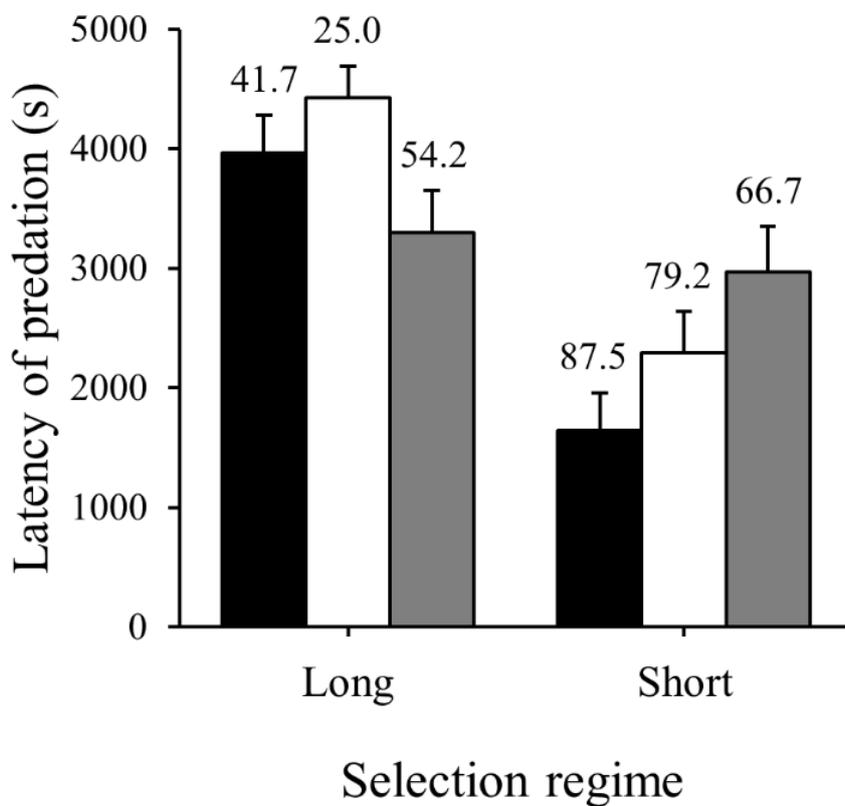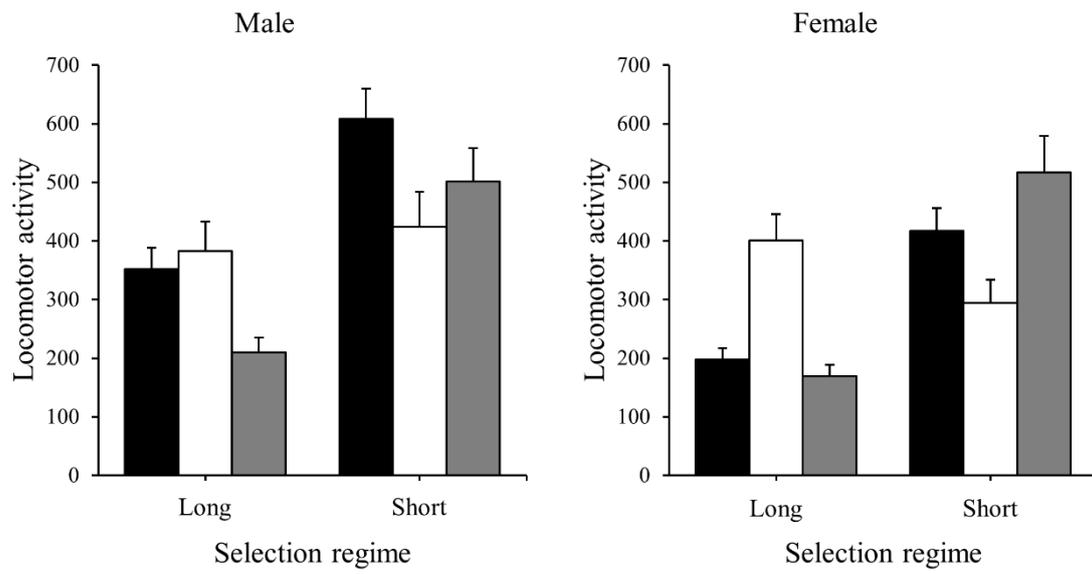
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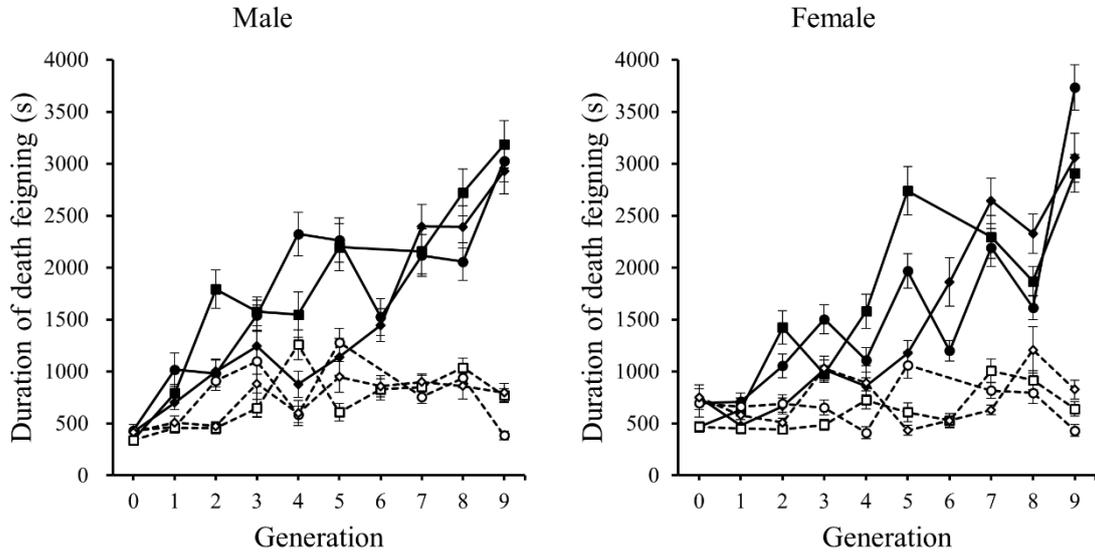


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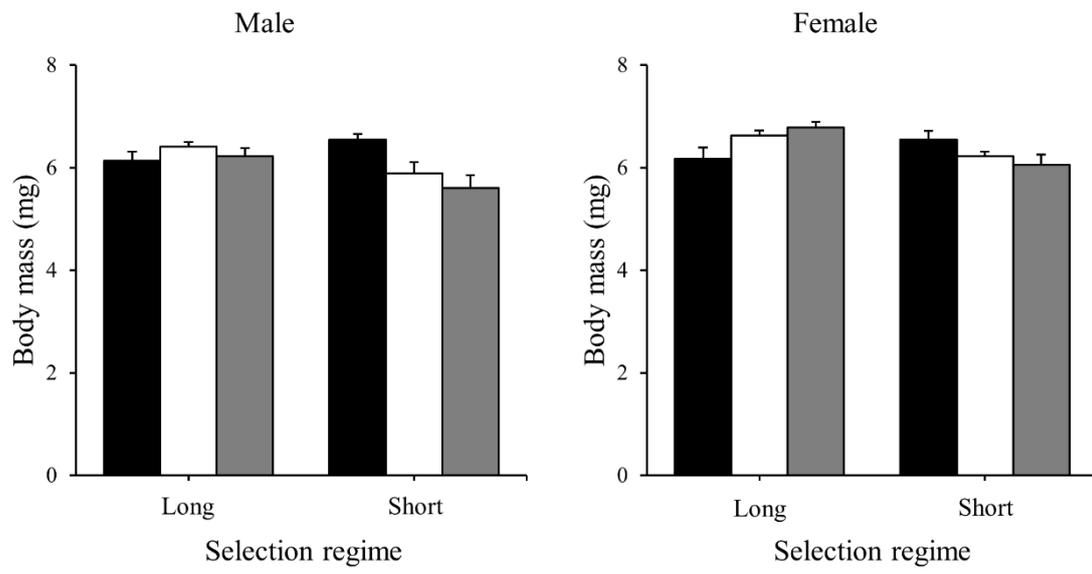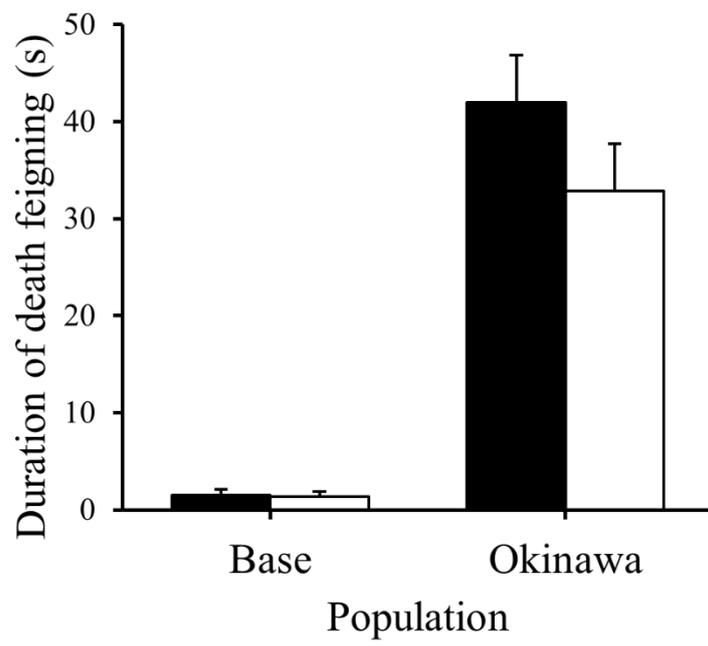543    Figure 1

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